Organic & Biomolecular Chemistry

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In vitro evolution of a Friedel–Crafts deoxyribozyme†

Cite this: DOI: 10.1039/c3ob40080h

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Received 15th January 2013,
Accepted 15th February 2013

DOI: 10.1039/c3ob40080h

www.rsc.org/obc

We report the *in vitro* selection of a single-stranded 72-nucleotide DNA enzyme (deoxyribozyme) that catalyzes a Friedel–Crafts reaction between an indole and acyl imidazole in good yield and in aqueous solvent. Appreciable Friedel–Crafts product requires addition of copper nitrate and the deoxyribozyme. We observe deoxyribozyme-mediated bond formation for both *in cis* and *in trans* Friedel–Crafts reactions.

Well-folded single-stranded RNAs and DNAs can achieve structural diversities and complexities that approach those observed in globular proteins.[1] Like proteins, single-stranded RNAs and DNAs can have enzymatic activity. A relatively large number of naturally occurring and unnatural RNA enzymes (ribozymes) are known.[2] In contrast, a much smaller number of DNA enzymes (deoxyribozymes) have been reported.[3–5] In comparison to ribozymes, deoxyribozymes are relatively stable and inexpensive to prepare, making these DNAs potentially useful as catalysts. A modest number of deoxyribozymes have been found that catalyze bond-forming or bond-breaking reactions involving nucleic acids, including ion-dependent RNA cleavage,[6,7] DNA cleavage,[8] DNA phosphorylation,[9] DNA adenylation,[10] DNA deglycosylation,[11] and thymine dimer photoreversion[12] have been reported. A smaller subset of deoxyribozymes have been reported to facilitate intramolecular or intermolecular reactions that expand beyond nucleic acid chemistry.[13]

Like a protein enzyme, a single-stranded DNA (ssDNA) deoxyribozyme would be expected to fold into a precise three-dimensional shape, directly engage with reactant(s), stabilize a reaction transition state complex in a well-defined catalytic pocket, and potentially participate in catalysis through the use of, or combination of, general acid-, general base-, Lewis acid-, or hydrophobic effect-dependent mechanisms (Fig. 1A).

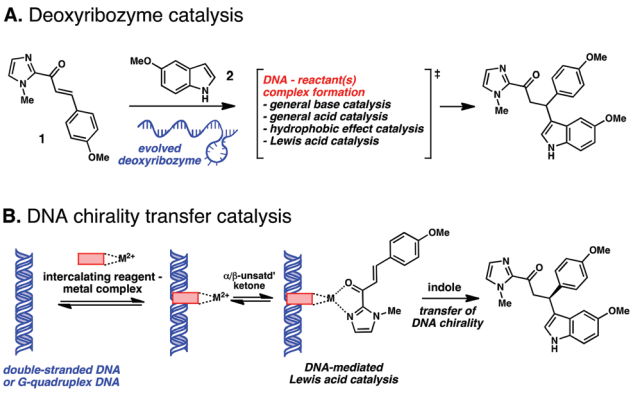

Fig. 1 Friedel–Crafts reactions *via*: (A) single-stranded deoxyribozyme-mediated bond formation. A folded single-stranded DNA engages directly with reactant(s) and stabilizes a reaction transition state, thereby facilitating bond formation. (B) dsDNA chirality transfer catalysis. Double-stranded DNA, or G-quadruplex DNA participates in the formation of a DNA – chelating metal reagent – reactant complex, thereby facilitating transfer of DNA stereochemistry to the resulting product. M = metal.

An alternative approach to facilitating organic reactions using DNA is DNA-based chirality transfer catalysis.[14,15] In this approach, DNA alone doesn't necessarily bind reactants, or stabilize a transition state through the formation of a DNA-reactant(s) complex. A hybrid catalyst is first generated by pre-complexing double-stranded DNA (dsDNA) or G-quadruplex DNA with a DNA-intercalating transition-metal reagent. Together, these components permit catalysis (such as Lewis acid-mediated catalysis), as well as the transfer of chirality from the DNA double helix to the stereocenter(s) formed in the reaction. DNA-based asymmetric catalysis has recently been applied to a copper(II)-catalyzed Friedel–Crafts reaction (Fig. 1B).[16]

While DNA chirality transfer chemistry will undoubtedly continue to be a useful method to impart stereocontrol, the development of single-stranded deoxyribozyme catalysts that directly participate in facilitating a chemical transformation is

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†Electronic supplementary information (ESI) available: Experimental details and characterization of new compounds. See DOI: 10.1039/c3ob40080h

needed to expand the repertoire of deoxyribozyme catalysis, and their study may lead to a deeper understanding of catalysis mechanisms. Inspired by recent work using double-stranded DNA[16] or G-quadruplex DNA[17] as a reagent to impart stereocontrol for Friedel–Crafts reactions, and as part of an effort to expand the synthetic scope of deoxyribozyme-mediated synthesis, we set out to evolve a deoxyribozyme for the Friedel–Crafts reaction between acyl imidazole **1** and 5-methoxy indole **2** (Fig. 1A).

Initially, we attempted to isolate deoxyribozymes using an *in vitro* selection scheme based on a biotin pull-down. We envisaged that a single stranded DNA library equipped with a 5′-linked acyl imidazole could be incubated with biotinylated indole **5**, and DNAs that form a covalent bond with **5** could be isolated *via* streptavidin-coated magnetic beads. DNAs covalently bonded to biotin could then be amplified by polymerase chain reaction (PCR). Toward this end, we synthesized bromo amide acyl imidazole **3**, and conjugated this compound to a 5′-thiol DNA primer (thiol-5′-GGAGCTCGCTTGTCG-3′) in quantitative yield to generate **2**, which was purified by PAGE gel electrophoresis and ethanol precipitation (Fig. 2). The conjugation product **4** was confirmed by matrix-assisted laser desorption/ionization mass spectrometry (ESI†). A single stranded DNA library was generated by polymerase chain reaction (PCR) on a 5′-CCTCGAGCGAACAGC-N_{40}-AGCTGATCCTGTGATGG-3′ DNA library template, using forward primer **4** and a reverse primer containing a 5′-$(PEG)_{18}$ spacer and $(AAC)_4$ terminus ($(AAC)_4(PEG)_{18}$-CCATCAGGATCAGCT-3′)). Since the reverse primer contains a 5′-$(AAC)_4(PEG)_{18}$-terminus, the two DNA strands of the PCR product have different molecular weights. In a manner that is similar to previous reports,[18] we were able to separate the single-stranded DNA library containing the 5′-linked acyl imidazole reactant from its complementary strand by denaturing PAGE gel electrophoresis.

We initially performed multiple rounds of selection for DNA-catalyzed bond formation by incubating the 5′-linked acyl imidazole DNA library with biotinylated indole **5** (Fig. 3), followed by pull-down of biotin-linked DNA on streptavidin-coated magnetic beads (Invitrogen) and PCR amplification of enriched DNAs. However, we observed high levels of DNA binding to the streptavidin-coated beads, and were unable to fully remove bead-binding DNAs from the library, even when negative selections to remove these DNAs were performed in advance of positive selections for bond formation, and extensive washing protocols. As a result, we were unable to selectively enrich and amplify active DNA using a streptavidin pull-down approach.

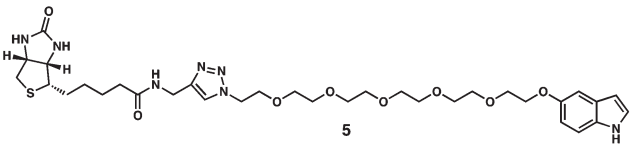

Fig. 3 Biotinylated indole **5**, which was initially used in streptavidin pull-down selections, then used as a co-reactant during gel-shift-based selections.

Since biotinylated indole **5** has a large molecular weight (703 Da), we were able to employ an alternative selection strategy to enrich active DNAs that is based on PAGE gel-shift of active DNAs. Each selection round consisted of three interacted steps: (1) incubating the 5′-linked acyl imidazole DNA library with **5**; (2) denaturing PAGE separation of active (higher molecular weight) DNA strands; and (3) PCR amplification and denaturing PAGE gel electrophoresis to amplify and isolate acyl imidazole 5′-linked single-stranded DNA, respectively, which is enriched with active species (Fig. 4A). In each round, selection conditions consisted of 150 pmol (1.5 μM) DNA, 20 mM MOPS buffer (pH 6.5), 150 μM $Cu(NO_3)_2$, 400 nmol (400 μM) biotinylated indole **5**. Importantly, when we mixed 1.5 μM acyl imidazole **1**, 400 μM 5-methoxy indole **2**, and 150 μM $Cu(NO_3)_2$, but no DNA, in MOPS buffer for 24 hours at 25 °C, we did not observe any Friedel–Crafts product. Therefore, in our selection, only DNAs that actively facilitate bond formation between the DNA-linked acyl imidazole and biotin-indole **5** are enriched. Before PAGE analysis, the mixture is heated to 94 °C for 5 minutes, then analyzed following

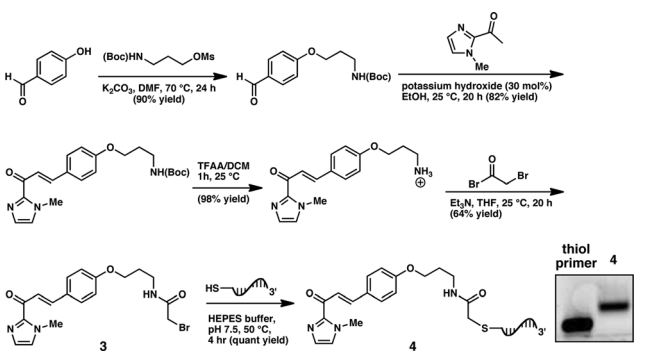

Fig. 2 Synthesis of bromoamide **3**, and α/β-unsaturated ketone-linked DNA primer **4**.

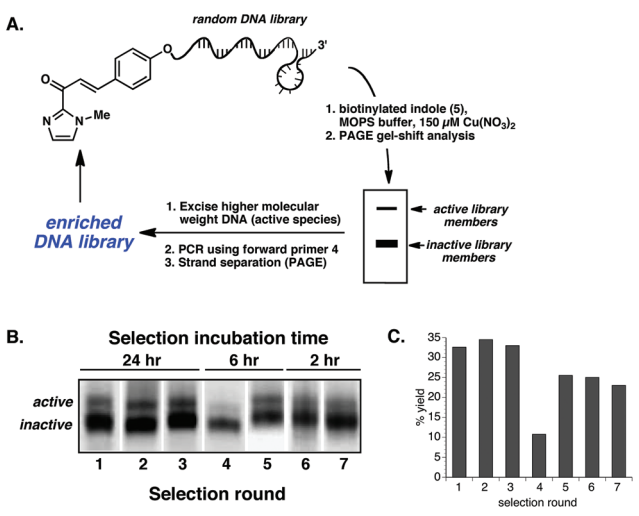

Fig. 4 (A) Gel-shift selection strategy to evolve catalytically active DNAs. (B) Gel-shift data from selections rounds 1–7. For each selection round, the ratio of active : inactive (higher molecular weight : lower molecular weight) DNA is provided in (C).

denaturing PAGE electrophoresis. Taken together, the heat denaturation and denaturing PAGE electrophoresis likely dissociates tightly bound DNA – **5** complexes. As a result, higher molecular weight DNAs should be covalently bound to **5**.

We performed three consecutive selection rounds with an incubation time of 24 h. This allowed us to enrich catalytic DNAs under relatively unstringent conditions (long reaction time). These selections were followed by two selection rounds with 6 hour incubation, and finally two rounds with a 2 hour incubation period. This allowed us to enrich those deoxyribozymes with the most robust activities. Following the final selection round, enriched DNAs were amplified by PCR, cloned into a pUC plasmid, and transformed into *E. coli*. Single clones were isolated and the enriched DNAs were sequenced using standard methods. We screened 20 clones for *in cis* reactivity, which was measured using a PAGE gel-shift assay that is identical to selection conditions. Interestingly, we observed very little sequence homology among the enriched DNAs we independently screened.

The most active DNA we isolated from our selection is referred to as M14 throughout. M14 is predicted to have a low folding energy (−16.2 kcal mol^{-1}), and contain an extended centralized base-paired sequence, which is flanked by ordered regions that contain multiple stem-loops and higher ordered structures (ESI†). As stated previously, running the Friedel–Crafts reaction under selection conditions (25 °C, 150 μM $Cu(NO_3)_2$, 1.5 μM acyl imidazole **1** and 400 μM biotin indole **5**) did not yield appreciable product (Table 1, entry 1). However, the *in cis* reaction using acyl imidazole-linked M14 DNA generated 32% product, as determined by gel-shift (Table 1, entry 2). Bond formation requires addition of Cu^{2+}. Running the reaction without $Cu(NO_3)_2$ did not generate appreciable levels of higher molecular weight DNA (Table 1, entry 3).

We next determined if clone M14 could facilitate a Friedel–Crafts reaction *in trans*, wherein neither reactant is tethered to the DNA. When we reacted 2 mM acyl imidazole **1**, 10 mM indole **2**, 0.6 mM $Cu(NO_3)_2$, and 1 mM M14 DNA at 25 °C for 24 hours, we observed the formation of the expected Friedel–Crafts product in 72% yield (Table 1, entry 4). Lowering the amount of M14 DNA dramatically lowered reaction yield. For example, using the same conditions, but 10 mol% M14 DNA, resulted in 18% yield of the Friedel–Crafts product (Table 1, entry 5). Performing the reaction in the absence of DNA, or in the absence of $Cu(NO_3)_2$, resulted in ≤10% yield of the Friedel–Crafts product (Table 1, entries 6 and 7, respectively), which is consistent with previous reports.[16,17] Affinity constants for the interaction between M14 DNA and reactants, as well as the molecular mechanisms of catalysis, are currently unknown. However, the increase in product yield for the *in trans* reaction, compared to the *in cis* reaction, is likely a result, at least in part, of the increased concentration of reactants. Since the selection is designed to enrich DNA that mediates a single bond forming reaction, and does not select for high catalyst turnover, it is unsurprising that relatively high amounts of deoxyribozyme are needed to observe good product yield. The precise sequence of M14 is required to achieve good reaction yield. An *in trans* reaction using a DNA sequence different from M14 does not generate appreciable levels of Friedel–Crafts product (Table 1, entry 8).

The enantioselectivity of M14-catalyzed reactions was not determined experimentally. However, good stereoselectivity in DNA-catalyzed[13] and DNA-templated[19] reactions has previously been demonstrated.

In summary, we have used a gel-shift *in vitro* nucleic acid selection to identify a 72-nucleotide deoxyribozyme that catalyzes a Friedel–Crafts reaction This deoxyribozyme functions well in the *in cis* reaction, wherein the acyl imidazole moiety is tethered the 5′ end of DNA and the indole is liked to a biotin moiety at position 5. When the reaction is run *in trans*, at a higher concentration (to facilitate isolation and characterization of product), the deoxyribozyme catalyzes formation of the Friedel–Crafts product in 72% isolated yield. Deoxyribozyme activity is dependent upon addition of Cu^{2+}; no appreciable yield is observed in the absence of $Cu(NO_3)_2$. Since appreciable yield of Friedel–Crafts product is dependent upon M14 DNA, and appreciable levels of product is not observed when a random DNA sequence is used, the mechanism of enzymatic action likely differs from DNA chirality transfer catalysis. Our ongoing efforts seek to understand the molecular mechanisms of M14 deoxyribozyme catalysis.

Table 1 *In trans* and *in cis* Friedel–Crafts reactions catalyzed by M14 DNA

Entry	*In cis* or *in trans*	Catalyst	Conversion (%)
1[a]	*In cis*	No	0
2	*In cis*	M14-Cu	32
3	*In cis*	M14	0
4[b]	*In trans*	M14-Cu (50 mol%)	72
5	*In trans*	M14-Cu (10 mol%)	18
6	*In trans*	M14	10
7	*In trans*	No	7
8	*In trans*	Random DNA	5

[a] Reaction conditions: MOPS buffer, pH = 6.5, 150 μM $Cu(NO_3)_2$, 1.5 μM acyl imidazole-linked DNA, 400 μM biotin indole 5, 25 °C, 24 h. [b] Reaction conditions: MOPS buffer, pH = 6.5, 0.6 mM $Cu(NO_3)_2$, 2 mM **1**, 10 mM **2**, 1 mM M14 DNA, for entry 4, and 0.2 mM M14 DNA for entry 5, 25 °C, 24 h. Cu = copper.

Notes and references

1 K. Y. Chang and G. Varani, *Nat. Struct. Biol.*, 1997, **4**, 854–858.
2 E. A. Doherty and J. A. Doudna, *Annu. Rev. Biochem.*, 2000, **69**, 597–615.
3 S. K. Silverman, *Chem. Commun.*, 2008, 3467–3485.
4 Y. Li and R. R. Breaker, *Curr. Opin. Struct. Biol.*, 1999, **9**, 315–323.
5 S. K. Silverman, *Org. Biomol. Chem.*, 2004, **2**, 2701–2706.
6 J. Li, W. Zheng, A. H. Kwon and Y. Lu, *Nucleic Acids Res.*, 2000, **28**, 481–488.
7 S. K. Silverman, *Nucleic Acids Res.*, 2005, **33**, 6151–6163.

8 M. Chandra, A. Sachdeva and S. K. Silverman, *Nat. Chem. Biol.*, 2009, **5**, 718–720.
9 Y. Li and R. R. Breaker, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 2746–2751.
10 Y. Li, Y. Liu and R. R. Breaker, *Biochemistry*, 2000, **39**, 3106–3114.
11 T. L. Sheppard, P. Ordoukhanian and G. F. Joyce, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 7802–7807.
12 D. J. Chinnapen and D. Sen, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 65–69.
13 S. K. Silverman, *Angew. Chem., Int. Ed. Engl.*, 2010, **49**, 7180–7201.
14 G. Roelfes, A. J. Boersma and B. L. Feringa, *Chem. Commun.*, 2006, 635–637.
15 G. Roelfes and B. L. Feringa, *Angew. Chem., Int. Ed. Engl.*, 2005, **44**, 3230–3232.
16 A. J. Boersma, B. L. Feringa and G. Roelfes, *Angew. Chem., Int. Ed. Engl.*, 2009, **48**, 3346–3348.
17 C. Wang, Y. Li, G. Jia, Y. Liu, S. Lu and C. Li, *Chem. Commun.*, 2012, **48**, 6232–6234.
18 M. Chandra and S. K. Silverman, *J. Am. Chem. Soc.*, 2008, **130**, 2936–2937.
19 X. Y. Li and D. R. Liu, *J. Am. Chem. Soc.*, 2003, **125**, 10188–10189.

